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Peritoneal dialysis solution induces apoptosis of mesothelial cells

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Peritoneal dialysis solution induces apoptosis of mesothelial cells. For better simulation of the long-dwell exchanges in conventional CAPD, we have developed a modified mesothelial cell culture system consisting of a Transwell culture apparatus. The equilibration patterns of pH, dextrose and osmolality in the present culture system were observed to be very similar to those in human CAPD. The effects of six different peritoneal dialysis solutions on the apoptosis of mesothelial cells were evaluated using this modified culture system. The results imply that peritoneal dialysis solution *per se* may incite apoptosis of mesothelial cells, and also that low calcium peritoneal dialysis solution is a milder apoptosis stimulant as compared to the conventional peritoneal dialysis solution. Moreover, varying concentrations of dextrose in the peritoneal dialysis solution were not observed to significantly affect the apoptosis rate. The roles of ambient high concentrations of calcium and dextrose, low pH, as well as high osmolality in the apoptosis are also discussed.

Conventional peritoneal dialysis solutions that are used for CAPD are characterized by an unphysiologically low pH as well as high osmolality, glucose and calcium. Extensive studies have been carried out to investigate the importance as well as the effects of their biocompatibility, however, these studies have provoked much debate [1–3]. The validity of many *in vitro* studies that used cultured mesothelial cells or other types of cells is being questioned since these experimental models lacked many *in vivo* characteristics of CAPD [3]. For example, the lack of peritoneal equilibration system in cell culture would result in a persistence of adverse effects of dialysis solution during the dwell period, and thus would exaggerate the toxic effects of dialysis solution. This has been clearly demonstrated in two reports that revealed that mesothelial cells, which were maintained in a conventional single chamber culture system, were unable to survive for more than 40 minutes when the culture medium was abruptly replaced by peritoneal dialysis solution [4, 5]. Hence, for better simulation of the long-dwell exchanges in conventional CAPD, we have developed a modified mesothelial cell culture system consisting of a Transwell culture apparatus. The equilibration patterns of pH, dextrose and osmolality in the present culture system were observed to be very similar to those in human CAPD [6–8].

While it is apparent that apoptosis is a gene-controlled cell disposal process and its expression requires considerable time for activation of corresponding genes, it is clear that conventional single chamber culture systems, which only allow a very brief survival of mesothelial cells in dialysis solution, are not suitable for investigation of this particular type of cell change. The present success in establishing long dwell exchanges in a Transwell culture system thus provided a more suitable system to assess this specific type of cell death with no interference from the corresponding massive cell degeneration.

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Methods

Preparation and characterization of human mesothelial cells

Mesenteric or omental human tissues were obtained from consenting non-uremic patients undergoing elective abdominal surgery. The excised peritoneal tissue was trimmed to an appropriate size and stretched on sterile culture dishes. Sterile plastic cylinders of various diameters (2 to 5 cm) were placed atop the exposed mesothelial layers and firmly fixed with sterile rubber bands to prevent leakage and contamination of stromal cells. The mesothelial surfaces in plastic cylinder were first cleaned by washing with PBS 37°C, following which the enzyme solution (5% collagenase in RPMI-1640, Sigma) was immediately added to plastic cylinders, and they were further incubated for 75 minutes at 37°C in a humidified culture chamber. The digested mesothelial surfaces were then gently scraped with a plastic cell scraper (Costar) to release the surface cells. The digestion fluid was subsequently transferred to conical tubes and the cells were collected by centrifugation at 1000 × g for 10 minutes. After rinsing the cells with plain culture medium (RPMI-1640; Sigma), the cells were resuspended in growth medium (RPMI-1640) supplemented with 20% fetal bovine serum (Gibco), insulin (0.5 mg/ml; Sigma), transferin (0.5 mg/ml; Sigma), selineum (0.5 mg/ml; Merk), penicillin (100 U/ml; Gibco) and streptomycin (100 mg/ml; Gibco), and were further plated onto gelatin-coated culture flasks. Once the majority of mesothelial cells were attached to culture substrate, the culture flasks were gently rinsed with fresh growth medium to remove any unattached cells. The cells were then propagated to obtain 90% confluence prior to being harvested or subcultured. The harvested cells were then frozen for further use. In all experiments, mesothelial cells were thawed and pooled from stocks of several donors.

The mesothelial cells utilized for immunohistochemical characterization were subcultured onto slide flasks (Nuck). These slides were then fixed in 4% phosphate-buffered paraformaldehyde, pH 7.4, and stained for cytokeratin, vimentin, UEA-I and factor-VIII by using the immunofluorescent method. All cultured cells were observed to be strong positive for both cytokeratin and vimentin,

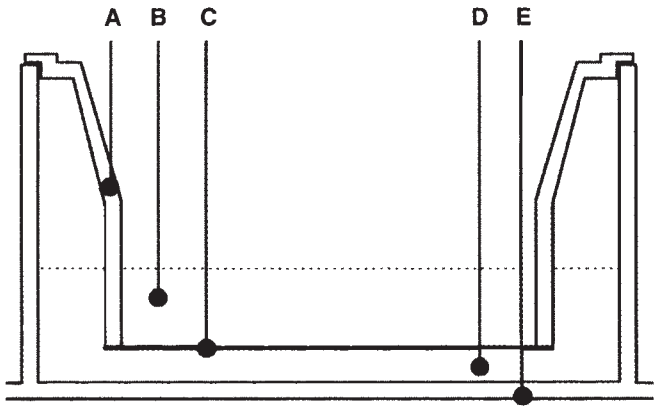

Fig. 1. *Cross section of a single Transwell.* **A.** Transwell insert. **B.** Upper reservoir for growth medium or dialysis solution. **C.** Microporous membrane. **D.** Lower reservoir for growth medium. **E.** Six-well cluster plate.

yet negative for endothelial cells markers (UEA-I and factor-VIII) (micrographs not shown).

Culture of human mesothelial cells on Transwell for in vitro dialysate treatment

The culture vessel used in the present dialysis experiments was a transparent, microporous membrane filter culture device (Transwell-Clear 3452, 24.5 mm diameter, membrane thickness 10 μm, pore size 3.0 μm; Costar), designed for use with six well cluster dish (Costar 3406). The growth media (3.1 ml) was first added to the cluster plate wells (lower reservoir) followed by the addition of Transwells (2 ml, upper reservoir; Fig. 1). After an initial equilibration period of four hours, the mesothelial cells were seeded at a high density (5×10^5 cells/well) into the upper reservoir of Transwells. The microporous membrane of Transwell was completely sealed by mesothelial monolayer within 24 hours. Prior to the initiation of *in vitro* dialysis, any unattached cells were removed by rinsing the Transwells with plain culture medium, and the sealing of the microporous membrane was further rechecked by examining the underside of this membrane for leakage of culture medium.

The dialysate treatment of mesothelial cells was designed in accordance with Popvich et al's original protocol of CAPD, that is, five changes in dialysate with four four-hour dwells during the daytime and one eight-hour overnight dwell [9]. The total dwell time in the present experiment was 48 hours. Furthermore, six different dialysates were used: Dianeal PD-2 and Dianeal Low Calcium peritoneal dialysis solutions containing either 1.5, 2.5 or 4.25% dextrose hydrous (Baxter Healthcare Corporation). The compositions of Dianeal PD-2 dialysate included dextrose hydrous (d-glucopyranose monohydrate) 1.5, 2.5 or 4.25 g/100 ml, sodium chloride 538 mg/100 ml, sodium lactate 448 mg/100 ml, calcium chloride dihydrate 25.7 mg/100 ml, and magnesium chloride hexahydrate 5.08 mg/100 ml. The compositions of Dianeal Low Calcium were same as those of Dianeal PD-2, with the exception that the amount of calcium chloride dihydrate was reduced to 18.3 mg/100 ml.

Morphological studies of cultured cells

For conducting transmission electron microscopic studies, the cells grown on plastic flasks or on Transwell membranes were first fixed with 2% phosphate-buffered glutaraldehyde and 1% osmic tetraoxide. These fixed cells were then subjected to *in situ* embedding procedure as has been previously described in the literature [10]. The resulting thin sections were then stained with uranyl acetate and lead citrate and viewed under a Zeiss electron microscope (EM10C), at 60 kV. In the case of scanning electron microscopic studies, the fixed cells were processed through the steps: dehydration, critical-point drying and gold coating as has been described previously in the literature [11]. The specimens were then viewed under a Hiatchi scanning electron microscope (S2300), at 25 kV.

Measurement of osmolality, pH and dextrose concentration

The concentration of dextrose in culture media or dialysate was determined by using an automated glucose/L-lactate analyzer (YSI Model 2300). Osmolality was determined by measuring the freezing-point depression or elevation of vapor pressure by using an osmometer (Advanced Instrument, Model 3MO). The pH was determined by using a pH meter (JENCO Model 6071) which was equipped with a combination pH electrode (Orion Model 8102).

Assessment of apoptosis of cultured mesothelial cells

Enzymatic *in situ* labeling of apoptosis induced DNA strand breaks was carried out by using a kit based on the TdT-mediated dUTP nick end labeling (TUNEL) method (In situ Cell Death Detection Kit #1684809; Fluorescein, Borhringer Mannheim, Germany) [12]. The mesothelial cells cultured on microporous membranes of Transwell cell culture chamber were first rinsed with phosphate buffered salin (PBS), then fixed with freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 minutes at room temperature and further incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for two minutes at 4°C. After rinsing with PBS, the membranes were removed form the buttom of Transwell and placed on glass slides. The microporous membranes were then incubated with freshly prepared TUNEL reaction mixture (100 μl/slide), which was prepared by mixing enzyme solution (terminal deoxynucleotidyl transferase from calf thymus, EC 2.7.7.31) and label solution (nucleotide mixture in reaction buffer) according to the manufacturer's instructions. In order to ensure a homogeneous spread of TUNEL reaction mixture across the microporous membrane and to avoid any evaporation losses, the membranes were covered with parafilm during the incubation. The membranes were then incubated in a humidified chamber (Immnuoloc StainPlate) for 60 minutes at 37°C. The reactions were terminated by rinsing the membranes three times with PBS. The microporous membranes were again placed on clean glass slides, mounted under glass coverslips and analyzed under fluorescence microscope (Olympus, BH2). The culture membranes which were used for negative controls were incubated in 100 μl/slide label solution without terminal transferase, instead of the TUNEL reaction mixture. The culture membranes used for positive controls were incubated with DNAse I (10 μg/ml) at room temperature for 10 minutes to induce DNA strand breaks prior to being incubated in TUNEL reaction mixture. The controls also included cultures that were

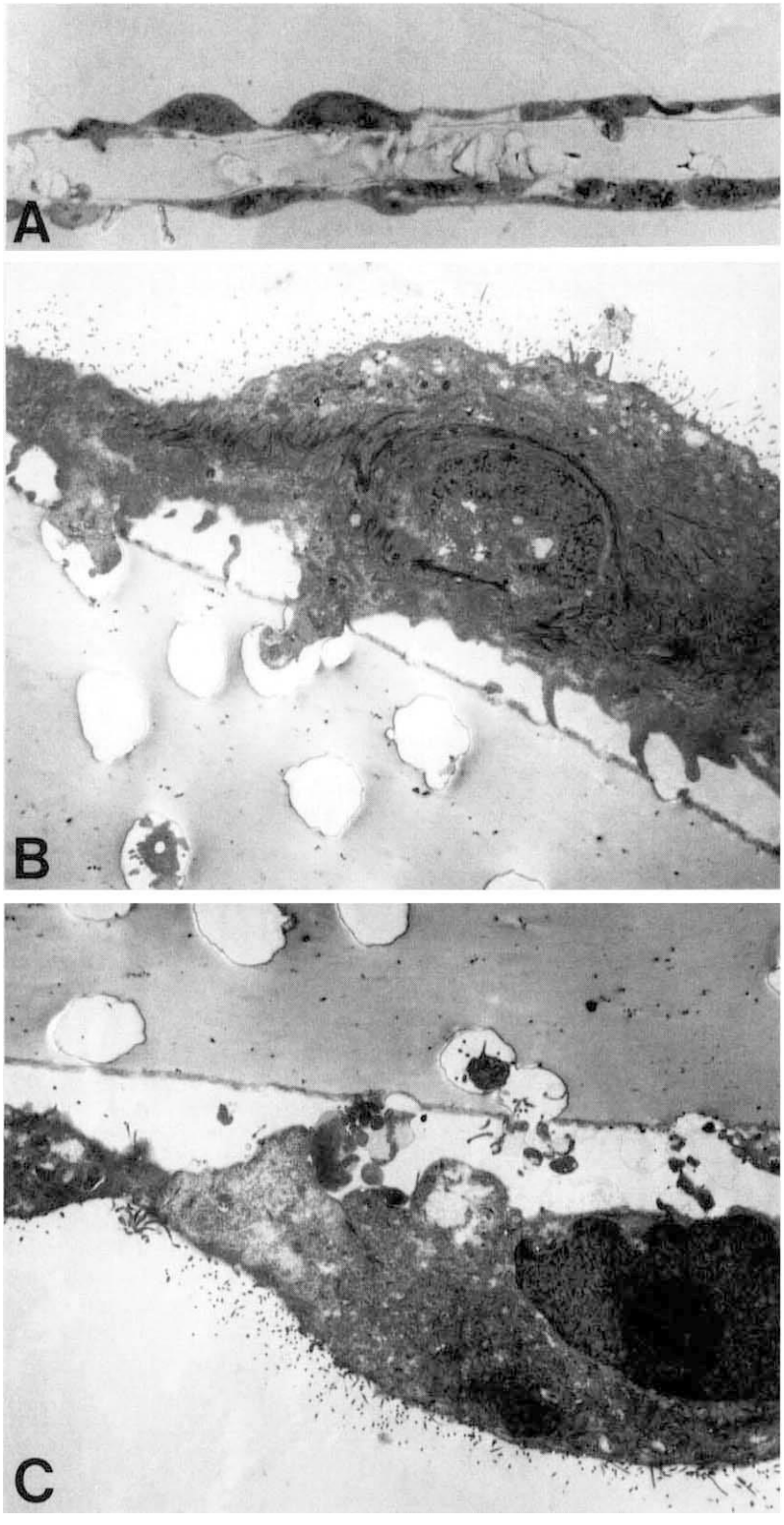

Fig. 2. *Vertical sections of initial mesothelial cells which were maintained on Transwell microporous membrane prior to their incubation with dialysis solution.* **A.** Thick section shows that both sides of membrane are paved with cells (toluidine blue stain, ×135). Cells facing upper reservoir (**B**) and lower reservoir (**C**) exhibit a characteristic morphology of mesothelial cells (B, C ×2876).

fed with growth medium in both the upper and lower chambers throughout the entire course of experiment.

The quantitative analysis of apoptosis of mesothelial cells was carried out by counting the number of TUNEL-positive nuclei observed in fluorescent micrographs taken under a ×20 objective lens. Five random micrographs were taken from each well. The labeling rate of positive control was almost 100%. The positive nuclear count per unit area thus represented total nuclear count

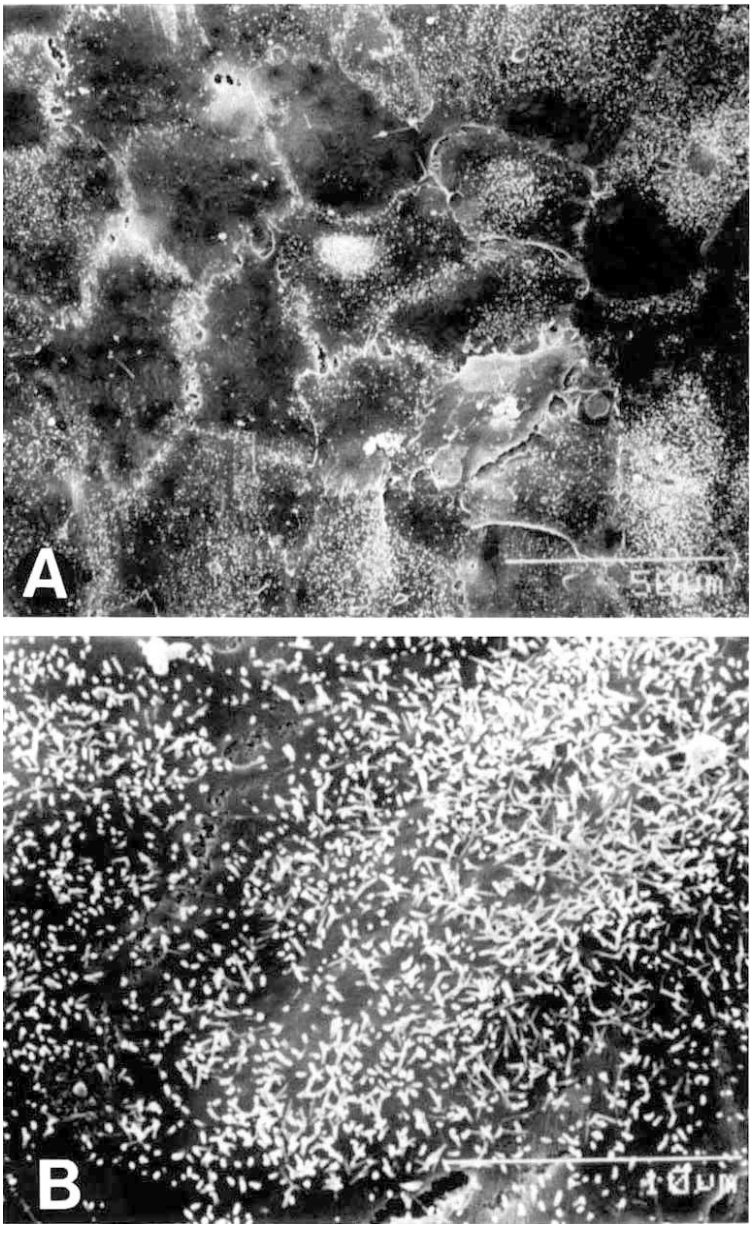

Fig. 3. *Scanning electron micrographs of initial mesothelial cell culture maintained on Transwell microporous membrane.* **A.** Lower magnification reveals the cobblestone appearance of mesothelial cells (scale mark = 50 μm). **B.** Higher magnification reveals abundant surface microvilli (scale mark = 10 μm).

per unit area. The apoptosis rate was defined as the ratio of dialysate-induced nuclear count to the DNAse I-induced nuclear count.

Results

Initial culture and characterization of mesothelial cells in the Transwell culture system

Figure 1 depicts the Transwell culture system. At the initial stage of preparation, when both the reservoirs of Transwell were loaded with growth medium, the human mesothelial cells that were grown on upper compartment migrated to the underside of microporous membrane, thereby forming a sandwich of monolayers that resulted in doubly sealing the membrane (Fig. 2A). These mesothelial cells, however, quickly resumed the cobblestone appearance and developed numerous surface microvilli at confluence (Fig. 3 A, B). The transmission electron microscopy of mesothelial cells on both sides of membrane revealed similar cellular structures characterized by the presence of abundant cytoplasmic microfilaments, numerous apical microvilli and pinocytoic vesicles, as well as a small amount of lamellar bodies (Fig. 2 B, C). Many channels of the microporous membrane were filled by mesothelial cell processes (Fig. 2 B, C).

Equilibration of peritoneal dialysis solutions in Transwells

Figure 4 depicts the serial monitoring of pH equilibration during dwell time in Transwell culture system. The initial pH of growth medium was 7.45 $\pm$ 0.5 while that of dialysate was 5.1 $\pm$ 0.2 at 37. The pH of dialysates in upper reservoir was observed to rise quickly to approximately 7.0 at about 60 minutes of the dwell period and then gradually approached equilibrium with that of

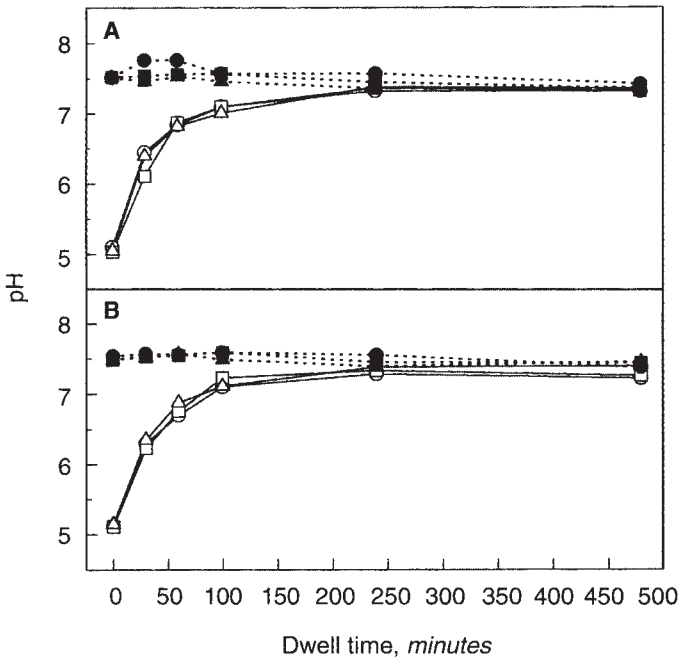

Fig. 4. *Equilibration profiles of pH as a function of dwell time in Transwell.* **A.** Upper reservoirs containing conventional dialysate PD-2 with 1.5% (p1.5, ○), 2.5% (p2.5, □) and 4.5% (p4.5, △) dextrose. The corresponding growth medium in the lower reservoir is indicated by mp1.5 (●), mp2.5 (■) and mp4.5 (▲), respectively. **B.** Upper reservoirs containing low calcium dialysate with 1.5% (lp1.5, ○), 2.5% (lp2.5, □) and 4.5% (lp4.5, △) dextrose. The corresponding growth medium in lower reservoir is indicated by mlp1.5 (●), mlp2.5 (■) and mlp4.5 (▲), respectively. Data are expressed as the mean pH value, $N = 6$.

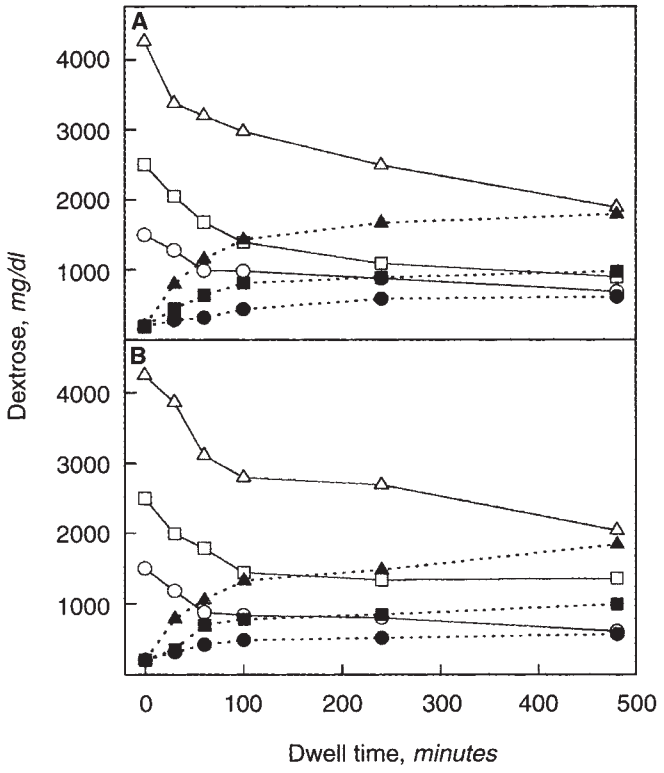

Fig. 5. *Equilibration profiles of dextrose as a function of dwell time in Transwell.* **A.** Upper reservoirs containing conventional dialysate PD-2. Symbols are: (○) p1.5; (□) p2.5; (△) p4.25; (●) mp1.5; (■) mp2.5; (▲) mp4.25. **B.** Upper reservoirs containing low calcium dialysate. Symbols are: (○) lp1.5; (□) lp2.5; (△) lp4.25; (●) mlp1.5; (■) mlp2.5; (▲) mlp4.25. Data are expressed as mean concentration of dextrose, $N = 6$; for curve labels, see Fig. 4.

growth medium in about 240 minutes of the dwell period (Fig. 4 A, B). The buffer capacity of growth medium was found to be well preserved during the long-dwell period (4 to 8 hr).

Measurements of dextrose concentration during the dwell period revealed a more rapid decline in dialysate dextrose concentration during the initial 100 minutes of dwell period (Fig. 5), followed by a gradual approach to equilibrium during the subsequent long-dwell period. The pattern of osmolality equilibration during dwell period was observed to correlate well with the dextrose equilibration pattern (Fig. 6).

No significant difference between the conventional dialysates and low calcium dialysates, in terms of the respective equilibration patterns of pH, dextrose, and osmolality, was observed.

Effects of peritoneal dialysate on the apoptosis of mesothelial cells

The apoptosis that was assessed with the help of an enzymatic *in situ* labeling technique (TUNEL) revealed that TUNEL-positive mesothelial cells had a bright nuclear fluorescence under fluorescent microscope (Fig. 7). The positive controls, after the deliberate treatment with DNAse I, showed labeling of all nuclei (labeling rate ≈ 100%; Fig. 7A), while the negative controls without incubation with terminal deoxynucleotidyl transferase showed an absence of nuclear labeling (not shown). The experimental controls that did not receive dialysate treatment also showed negative labeling by TUNEL assay (Fig. 7E). The TUNEL-positive cells in dialysate-treated cultures consisted of more compact nuclei as compared to those in pseudo-TUNEL-positive cells induced by DNAse I digestion (Fig. 7, B-D and F-H vs. A). To further verify the presence of apoptotic changes in mesothelial cells, we also examined the cells under a scanning electron microscope. The dialysate-treated monolayers of mesothelial cells consisted of scattered apoptotic cells with characteristic bud-like surface protuberances (Fig. 8). These cells also showed a shrinkage of cell body and loss of surface microvilli (Fig. 8).

The average apoptosis rate per unit area was observed to vary with the type of dialysate (Table 1). The mesothelial monolayers that were treated with low calcium dialysates exhibited lower apoptosis rate as compared to those treated with conventional dialysates (Student's *t*-test; all pairs of PD-2 vs. Dianeal low calcium, $P < 0.002$). The concentration of dextrose was observed to have no significant effect on the apoptosis rate (Student's *t*-test; all pairs of PD-2 with different dextrose concentrations, $P > 0.3$; all pairs of low calcium dialysates with different dextrose concentrations, $P > 0.08$).

Discussion

The present system allows a minimum of 10 dwell exchanges in a period of 48 hours without the occurrence of any widespread degeneration of mesothelial monolayers. The prolonged survival of mesothelial cells in our culture system may be attributed to the rapid equilibration of dialysis solution with subsequent reduction

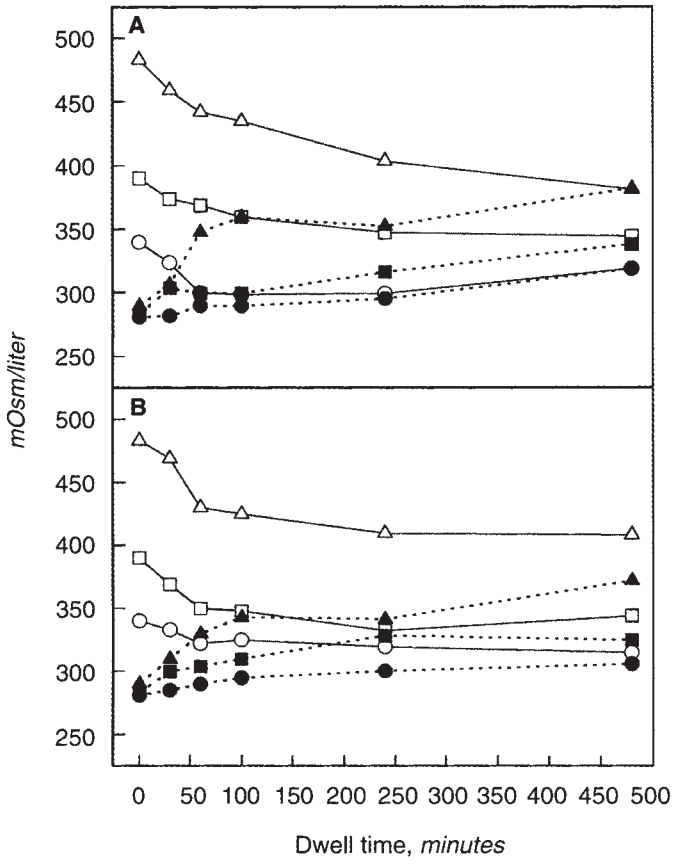

Fig. 6. *Equilibration profiles of osmolality as a function of dwell time in Transwell.* **A.** Upper reservoirs containing conventional dialysate PD-2. Symbols are: (○) p1.5; (□) p2.5; (△) p4.25; (●) mp1.5; (■) mp2.5; (▲) mp4.25. **B.** Upper reservoirs containing low calcium dialysate. Symbols are: (○) lp1.5; (□) lp2.5; (△) lp4.25; (●) mlp1.5; (■) mlp2.5; (▲) mlp4.25. Data are expressed as mean osmolality, $N = 6$; for curve labels, see Fig. 4.

of its inhibitory potential. Other mechanisms of mesothelial cells to maintain their integrity in our culture system might be similar to those in animal peritoneum during experimental peritoneal dialysis protocol as mentioned by Gotloib et al [13]. These mechanisms include a variety of adaptive changes such as increased cellular enzyme activity, intensified transmembrane active transport, higher rate of nutritional absorption and increased secretory activity [14].

The quantification of apoptosis by TUNEL method has several methodological considerations that are essential for the interpretation of experimental results. First of all, TUNEL may not label all the cells that are undergoing apoptotic change in a given population, because DNA fragmentation is a rather late event of apoptosis and not all apoptotic cells in a population are synchronized [15, 16]. Moreover, DNA fragmentation may take place even after cell elimination. In small intestine, only about 10% of the apoptotic cells exhibit DNA fragmentation while they are still a part of the tissue [17]. In case of the remaining apoptotic cells, this process takes place only after they are shed to the lumen [17]. However, whether this also occurs in case of the mesothelial cell culture is not yet known. Nevertheless, if it does, then the TUNEL assay would underestimate the apoptotic rate in the present experiments. Although the fraction of apoptotic cells that are detected by TUNEL may not be highly compatible with the real apoptotic rate, it is still the most practical way of assessing the quantity of apoptosis in a cell culture system. Furthermore, the TUNEL may also label even the necrotic cells containing fragmented DNA [16, 18, 19]. Hence, it is very important to validate the TUNEL assay by conducting supplementary morphological examination of TUNEL-positive cells. It has been reported earlier that TUNEL-positive necrotic cells lacked characteristic apoptotic morphology such as cell shrinkage, nuclear condensation or nuclear fragmentation [19]. These cells also demonstrated the uneven TUNEL staining predominantly found in the cytoplasm [19]. In the present study, the fact that TUNEL-positive mesothelial cells in dialysate-treated cultures consisted of more compact nuclei as compared to those in pseudo-TUNEL-positive cells induced by DNAse I digestion, along with clear nuclear staining without cytoplasmic diffusion, strongly supports the inference that these cells are true apoptotic cells. The scanning electron microscopy of dialysate-treated mesothelial cells offered further evidence of the existence of apoptosis by demonstrating the characteristics of cell shrinkage and surface protuberances.

Presently, there are no definite data indicating which factors in peritoneal dialysates may incite the apoptosis of mesothelial cells, and hence, further study is necessary to elucidate this phenomena. Several intrinsic factors of peritoneal dialysates such as calcium and magnesium concentration, pH, dextrose concentration and osmolality are worth discussing. Unphysiologically high calcium levels in conventional peritoneal dialysate is the most suspicious factor responsible for inciting it, since the present results revealed that mesothelial cells that were incubated with low calcium dialysate ($[Ca^{2+}]$, 1.245 mmol/liter) had a lower apoptotic rate as compared to conventional dialysate ($[Ca^{2+}]$, 1.748 mmol/liter). It has also been shown that the increase of Ca^{2+} concentration in the peritoneal dialysis solution caused a dose-dependent rise in the cytoplasmic Ca^{2+} in peritoneal cells [20]. Further, it is also well-established that modulation of intracellular Ca^{2+} plays a very important role in pathogenesis of cell injury and cell death [21]. The increase in intracellular Ca^{2+} may lead to activation of nuclear endonuclease as well as some apoptosis associated genes such as c-*fos*, c-*jun* and c-*myc* [21]. The involvement of Ca^{2+}-dependent endonuclease is supported by the fact that calcium chelation can protect these cells from apoptosis [22].

The role of pH in apoptosis is, however, not very clear. Both intracellular acidification and alkalinization have been reported to be responsible for the initiation of apoptosis in HL-60 cells [23, 24]. However, Zhu and Loh reported that the changes in cytosolic pH brought about by the modulation of extracellular pH did not initiate apoptosis, and concluded that a solitary change in cytosolic pH is not sufficient for initiating apoptosis in HL-60 cells [24].

A few studies have been carried out to investigate the effects of high ambient glucose and osmolality on the apoptosis of cells. Baumgartner-Parzer et al have demonstrated that incubation of human umbilical vein endothelial cells maintained in high glucose concentration (30 mmol/liter) for more than 48 hours increased the DNA fragmentation as compared to the cultures which were kept in low glucose concentration (5 mmol/liter) [25]. However, this effect has not been observed in some defined cell lines and fibroblasts. It has hence been speculated that the glucose-triggered apoptosis may be related to the DNA damage caused by glycation products of glyceraldehyde-3-phosphate and lysine [25, 26]. In the present experiments, the mesothelial cells that were

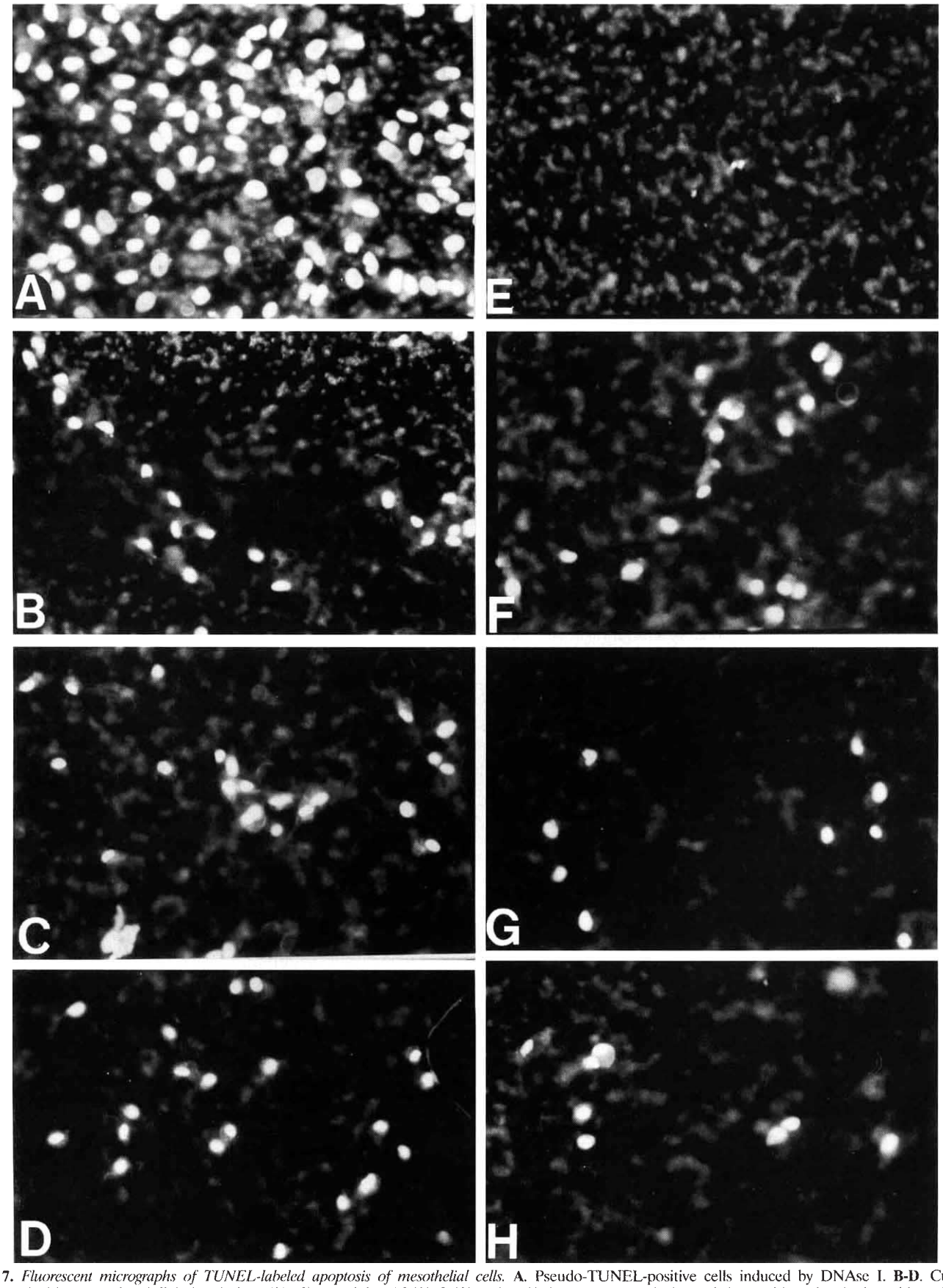

Fig. 7. *Fluorescent micrographs of TUNEL-labeled apoptosis of mesothelial cells.* **A.** Pseudo-TUNEL-positive cells induced by DNAse I. **B-D.** Cultures incubated with conventional dialysis solutions (PD-2) containing 4.25%, 2.5% and 1.5% dextrose, respectively. **E.** Culture without incubation with peritoneal dialysis. **F-H.** Cultures incubated with low calcium dialysis solutions containing 4.25%, 2.5% and 1.5% dextrose, respectively (all micrographs ×118).

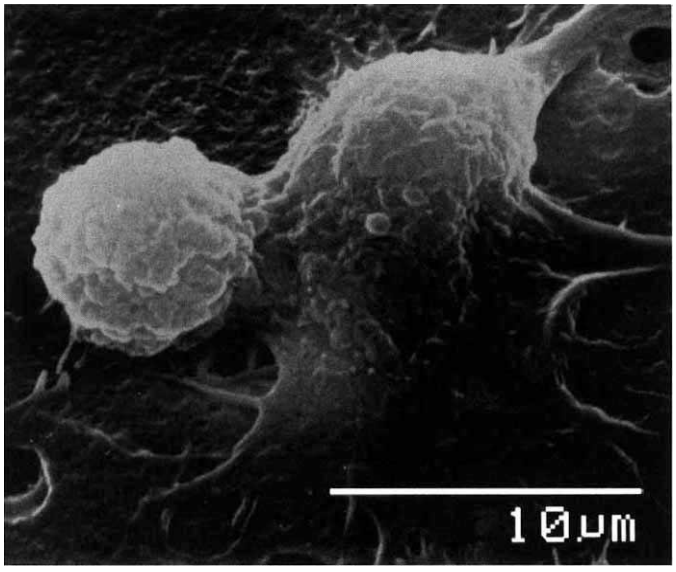

Fig. 8. *Scanning electron micrograph of a dialysate-treated mesothelial cell showing an apoptotic morphology along with budding-like surface protuberance (scale mark = 10 μm).*

Table 1. Average apoptosis rate of dialysate-treated mesothelial cells

	Dextrose Concentration		
	1.5%	2.5%	4.25%
Conventional dialysate $[Ca^{2+}]$: 1.748 mmol/liter	20.7 ± 1.5	21.5 ± 2.1	22.7 ± 2.0
Low calcium dialysate $[Ca^{2+}]$: 1.245 mmol/liter	9.6 ± 2.2	10.7 ± 2.7	11.8 ± 2.7

Data are expressed as mean apoptosis rate (%) ± SD (N = 6).

incubated with peritoneal dialysates were subjected to an even higher concentration of glucose (75.8 mmol/liter to 214.6 mmol/liter) and a longer incubation period (4 to 8 hr) as compared to that with Baumgartner-Parzer's experiments. It is hence quite possible that these mesothelial cells suffered the same glucose-triggered damage in human endothelial cells, despite the fact that no statistically significant difference in apoptotic rate was observed for dialysates containing different amounts of dextrose. Direct evidence of the effects of high osmolality *per se* on the apoptosis is, however, lacking. Nevertheless, the hypotonicity appears to be capable of altering the Ca^{2+}-dependent endonuclease activation and attenuation of the DNA damage by hydrogen peroxide [27].

In conclusion, the present study demonstrates the assessment of apoptosis of mesothelial cells in an *in vitro* system that simulated the conventional dwell procedure of peritoneal dialysis. The present results indicate that peritoneal dialysate *per se* might incite apoptosis of mesothelial cells and also that low calcium dialysate is a milder apoptosis stimulant as compared to conventional dialysate.

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